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13. ABSTRACT (Maximum 200 Words) This group has completed the goal set for <u>Task 1</u> ("To examine the immune responses to a model tumor antigen selectively expressed in DCs differentiated <i>in vivo</i> from transduced hematopoietic progenitor cells"). They have: 1) made lentiviral vectors expressing a model tumor antigen influenza hemagglutinin (HA); 2) developed a method to efficiently transduce mouse bone marrow progenitor cells that reconstitute immune and blood systems in irradiated and transplanted mice; 3) demonstrated that the transplanted and transduced cells can persistently present the HA model tumor antigen and stimulate T cell responses; 4) devised a tripartite strategies to mount powerful and specific attack on HA-expressing experimental tumors; and 5) demonstrated the efficacy to eradicate an aggressive pre-established tumor expressing the HA antigen. This study provides a proof-of-principle in immune competent animals models that transplantation of antigen gene transduced hematopoietic progenitor cells adds a new component to achieve potent and sustained antigen-specific immunotherapy of aggressive tumors. Key discoveries by this group and their Hopkins colleagues were published in a leading biomedical research journal <i>Nature Medicine</i> (10: 882).		
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Introduction

This is the final report for a New Investigator Award (DAMD17-02-1-0075) with the proposal entitled "Antigen specific immunotherapy using lentivirus transduced hematopoietic progenitor cells: a novel approach for the treatment of metastatic prostate cancer".

Body

Background: Over forty thousand Americans die annually from metastatic prostate cancers even after extensive treatments, and recent data suggested that the death rate was underestimated. Standard chemo- and radiation- therapies have been ineffective at prolonging the survival of these patients and are often associated with severe side effects. New forms of therapies therefore are urgently needed for these dying patients. Biological therapies, particularly immunotherapy which boosts the body's self defense against tumor cells, are increasingly used as adjunct or alternative treatments for prostate and other cancers. Many therapeutic tumor vaccination approaches have focused on engineering antigen presenting cells (APCs), particularly dendritic cells (DCs). However, recent studies using *ex vivo* antigen-loaded DCs as cancer vaccines have shown limited success.

Objective/ Hypothesis: We hypothesize that hematopoietic stem/progenitor cells (HSPCs) transduced with a specific tumor antigen gene followed by transplantation can be differentiated *in vivo* into functional DCs that express a tumor antigen at high levels. Large numbers of antigen-expressing DCs that mature *in vivo* can effectively activate tumor-specific T cells, resulting in more potent and long-lasting antitumor immunity. This novel approach to generate large numbers of antigen-specific DCs *in vivo* may overcome limitations observed in early DC-based clinical trials.

Specific aims: We will test whether these *in vivo* differentiated, antigen-expressing DCs maintain their functional capacity in priming antitumor immune responses and ultimately reverse immune tolerance to tumor antigens. Specifically, we will: 1) examine the immune responses to a specific model tumor antigen in mice with reconstituted immune system by HSPCs transduced with the antigen; 2) examine if the transduced DCs can generate immunity to prevent and eradicate the antigen-expressing tumor cells in mouse models; 3) examine if this approach can prevent and eradicate the tumor cells expressing prostate specific membrane antigen (PSMA) in a similar mouse model.

Study designs: In addition to PSMA, we will also use a well-characterized model tumor antigen, influenza hemagglutinin (HA), in order to track directly antigen-specific immune responses and quickly assess antitumor efficacy using transgenic mice expressing T cell receptors recognizing the HA antigen. This system allows us to precisely determine functional roles of HA-expressing DCs in inducing T cell activation *versus* tolerance, and define optimal vaccination strategies. HSPCs from mouse bone marrow will be transduced *ex vivo* with lentiviral vectors expressing PSMA or HA under the control of a DC-selective promoter, and then transplanted into syngeneic recipient mice. We will evaluate the efficacy and specificity of DCs differentiated from transduced HSPCs with either HA or PSMA gene in priming immune responses to eliminate implanted TRAMP-C2 mouse prostate tumor cells (engineered to expressing either HA or PSMA).

Relevance: These studies will serve to identify the best vaccination strategy that will subsequently be tested in clinical trials as a treatment for metastatic prostate cancer, targeting PSMA or other emerging prostate tumor antigens. Since PSMA is also over-expressed in the neovasculature of a variety of malignant neoplasms, this immune therapy approach may also apply to other cancers such as metastatic breast cancers.

Task 1: To examine the immune responses to a model tumor antigen selectively expressed in DCs differentiated *in vivo* from transduced HSPCs. A well-characterized model tumor antigen, influenza hemagglutinin (HA), along with transgenic mice (lines expressing T cell receptors recognizing the HA antigen and lines expressing HA in prostate epithelial cells), will be used. This system allows us to

precisely determine functional roles of HA-expressing DCs (differentiated *in vivo* from transduced HSPCs) in inducing T cell activation *versus* tolerance.

Since PCRP recommended and only funded the Task 1, we have been focusing on Task 1 in the past.

Key Research Accomplishments

1. We have successfully constructed lentiviral vectors expressing HA (the model tumor antigen used in this study) and several control vectors expressing GFP or NGFR (inactive nerve growth factor receptor). High titer virus stocks can be made. All the insert genes functioned as designed.
2. In addition to *human* hematopoietic stem/progenitor cells (HSPCs), we also found that the same approach can be applied to *mouse* HSPCs (Ref. 1-2). This is important because mouse models are used as pre-clinical studies in this proposal (Ref. 1-2). We devised the method to efficiently transduce isolated HSPCs from mouse bone marrow cells that are found to more difficult to be transduced by lentiviral vectors. In fact, the established lentivirus-mediated gene transduction technology allowed us to investigate a related approach to improve bone marrow transplantation (Ref. 3-4).
3. We utilized a lentivirus to transduce mouse HSPCs, then transplanted the modified cells and analyzed expression of the transgene in the dendritic cells (DCs) in relevant lymphoid compartments to determine the efficacy of this approach. HSPCs were transduced with GFP as a reporter gene under the control of a strong constitutive promoter (Ref. 1-2, 5). Transduction was followed by transplantation into lethally irradiated recipients. After engraftment, DCs were isolated from spleen and lymph nodes, and FACS analysis was performed to determine the percentage of DCs (CD11c⁺/MHC II^{high} cells) that expressed GFP. At 5 weeks post transplant, an average of 34% of the DC expressed the gene in the spleen and 25% in the LN (Fig. 1 in Ref. 5/Appendix 1). Thus, transduction of precursors, differentiation of transduced precursors into DCs, and trafficking of DCs to secondary lymphoid organs all were efficient. Similar percentages of transduced cells were obtained in the CD11c⁻ fraction (Fig. 1 in Appendix 1) as well as the lymphocyte fraction after collagenase digestion, indicating that transduction was not selective for DC progenitors. Similar numbers of splenocytes were obtained from non-transplanted and transplanted mice, with equivalent percentages of B and T lymphocytes, indicating that reconstitution is well underway at this time (not all data shown). The proportion of CD11c DCs expressing the transgene did not change after administration of agonist ant-CD40 antibody, indicating that systemic DC activation did not selectively affect transgene⁺ vs transgene⁻ DC progeny.
4. Using the HA as a model tumor antigen, we have examined specific antigen presentation and T cell activation in mice post *ex vivo* gene transduction (with HA gene) and transplantation of mouse HSPCs. Drs. Yan Cui, Drew Pardoll and I (co-investigators of this application), together with my colleagues at Johns Hopkins Oncology Center, performed large scales of tumor vaccinations and eradication experiments with HA-expressing experimental tumors. We found that 1) the transplanted and HA gene -transduced cells can persistently present the HA model tumor antigen and stimulate T cell responses; 2) a tripartite strategy is required to mount powerful and specific attack on HA-expressing experimental tumors; and 3) this combined approach can eradicate an aggressive pre-established tumor expressing the HA antigen. Details of immune analyses have been just published (Ref. 5) and included as Appendix 1.

Reportable outcomes

Manuscript: (Ref. 5)

Yan Cui, Erin Kelleher, Erin Straley, Ephraim Fuchs, Kevin Gorski, Hyam Levitsky, Ivan Borrello, Curt I. Civin, Stephen Schoenberger, Linzhao Cheng, Drew M. Pardoll, Katharine A. Whartenby.

Tumor immunotherapy with antigen transduced hematopoietic stem cells. **Nature Medicine**, 2003, 10:882.

Reagents:

1. Lentiviral vectors expressing the HA antigen and other reporter genes (GFP or NGFR)
2. Lentiviral vectors expressing the PSMA gene, used by Drs. Samuel Denmeade, John Issacs, Roberto Pili (currently PCRP awardees) at Johns Hopkins Oncology, and Dr. Party Pomper at Johns Hopkins University Radiology.

Methods:

1. Efficient and stable gene transfer into mouse HSPCs capable of reconstituting the blood and immune systems in irradiated recipient mice;
2. Efficient and sustained transgene expression in engrafted cells derived from transduced HSPCs, including dendritic cells that play a central role in modulating immune responses
3. A tripartite strategy (BMT with antigen-transduced HSPCs, expansion and activation of derived DCs in vivo by Flt3 and CD40 activators and donor lymphocyte infusion) to eradicate aggressive tumors.

Employment and research opportunities:

Dr. Yan Cui, a postdoctoral fellow co-mentored by Drs. Linzhao Cheng and Drew Pardoll, and a co-investigator of this application, is the first author of the paper cited in ref. 5. She has accepted an independent research position as **Assistant Professor in Louisiana State University Health Science Center, Dept. of Medicine, Gene Therapy Program**. She is planning to continue using gene therapy approach to develop new immunotherapies for aggressive cancers.

Conclusions

This is the first annual report for a New Investigator Award. We have made significant progress for Task 1 that was funded by PCRP as listed above. The funding (\$75,000, direct) has been crucial to support me to participate the team effort at Johns Hopkins Oncology Center and to develop novel cancer immuno-therapies. Together with other our Hopkins colleagues, Dr. Yan Cui, Drew Pardoll and I (co-investigators in this application). We have: 1) made lentiviral vectors expressing a model tumor antigen HA; 2) developed a method to efficiently transduce mouse bone marrow progenitor cells that reconstitute immune and blood systems in irradiated and transplanted mice; 3) demonstrated that the transplanted and transduced cells can persistently present the HA model tumor antigen and stimulate T cell responses; 4) devised a tripartite strategies to mount powerful and specific attack on HA-expressing experimental tumors; and 5) demonstrated the efficacy to eradicate an aggressive pre-established tumor expressing the HA antigen. This study provides a proof-of-principle in immune competent animals models that transplantation of antigen gene transduced hematopoietic progenitor cells adds a new component to achieve potent and sustained antigen-specific immunotherapy of aggressive tumors. Key discoveries by this group and their Hopkins colleagues are published in a leading medicine journal *Nature Medicine*.

A Note

My lab has shifted research focuses after we joined the Johns Hopkins Institute for Cell Engineering in Fall 2003. However, Dr. Drew Pardoll (a co-investigator of this proposal) and my other former colleagues at Johns Hopkins Oncology Center are continuing the effort of immuno-therapies to treat metastatic cancers including prostate cancers.

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Appendix: Ref. 5

Immunotherapy of established tumors using bone marrow transplantation with antigen gene-modified hematopoietic stem cells

Yan Cui^{1,3}, Erin Kelleher¹, Erin Straley¹, Ephraim Fuchs¹, Kevin Gorski¹, Hyam Levitsky¹, Ivan Borrello¹, Curt I Civin¹, Stephen P Schoenberger², Linzhao Cheng¹, Drew M Pardoll¹ & Katharine A Whartenby¹

A major focus of cancer immunotherapy is to develop strategies to induce T-cell responses through presentation of tumor antigens by dendritic cells (DCs). Current vaccines are limited in their ability to efficiently transfer antigens to DCs *in vivo*. *Ex vivo*-generated DCs can be efficiently loaded with antigen but after reinjection, few DCs traffic to secondary lymphoid organs, the critical sites for antigen presentation. To enhance efficiency and durability of antigen presentation by DCs, we transduced hematopoietic stem-progenitor cells (HSCs) with a model tumor antigen and then transplanted the gene-modified cells into irradiated recipient mice, which resulted in efficient expression of the transgene in a large proportion of donor derived DCs in lymphoid organs. The combination of bone marrow transplantation (BMT) using transduced HSCs, systemic agents that generate and activate DCs, and mature T-cell infusion resulted in substantial expansion and activation of antigen-specific T cells. This tripartite strategy provided potent antigen-specific immunotherapy for an aggressive established tumor.

A central goal of cancer immunotherapy is to activate tumor antigen-specific T cells. To enhance T-cell responses to tumors, DCs have been investigated for their ability to prime CD4⁺ and CD8⁺ T cells. Established techniques for growing DCs in culture *ex vivo* have allowed development of DC-based vaccines. In light of promising preclinical results, clinical trials for many tumor types^{1–9} have been initiated using *ex vivo*-generated DC vaccines. Although these trials showed overall that immune responses could be generated, long-term cures were not generally achieved. These results underscore both the potentials and limitations of this approach. One limitation of reinfused *ex vivo*-generated DCs is that relatively few DCs successfully traffic to spleen or draining lymph nodes¹⁰, and those that do can be rapidly cleared by host cytotoxic T lymphocytes (CTLs)¹¹.

To provide for increased expression of antigen by DCs *in vivo*, we developed a strategy of transduction of HSCs with genes encoding antigen, followed by transplantation. Introduction of antigen-encoding genes into the HSCs combines both effective delivery of antigen to the DC progenitors and the benefits of autologous BMT, which is currently an important treatment for a number of hematologic malignancies. Additionally, the post-transplant setting is an optimal time for redirecting the immune system toward specific tumor antigens^{12–15}.

Lentiviral vector technology allows efficient transduction of HSCs^{16,17}. Here we show that lentiviral transduction of HSCs results in expression of encoded antigen in a large proportion of lymphoid

organ DCs. In order to bypass the induction of central tolerance from repopulation of the thymus with antigen-expressing DCs, we followed the BMT with infusion of mature post-thymic lymphocytes, modeling autologous donor lymphocyte infusions (DLI). Finally, to increase the numbers and activation states of DCs, we administered systemic Flt-3 ligand (Flt-3L) and an activating antibody to CD40, as Flt-3L generates large numbers of DCs *in vivo*¹⁸ and CD40-mediated maturation of DCs enhances the protective effect of antitumor vaccines^{19,20}. In the present study, we show that this tripartite approach of bone marrow transduction, DLI and systemic DC modulation leads to dramatic expansion of antigen-specific T cells and successful treatment of an established tumor.

RESULTS

Expression of transgenes in DCs *in vivo*

Lentivirally transduced HSCs were transplanted into lethally irradiated recipients, which were then analyzed for transgene expression by the DCs in lymphoid compartments. HSCs were transduced with constitutively expressed green fluorescent protein (GFP) as a reporter gene. After engraftment, DCs were isolated from spleen and lymph nodes, and fluorescence-activated cell sorting (FACS) analysis was done to determine the percentage of DCs (CD11c⁺MHC II^{hi} cells) that expressed GFP. Five weeks after transplantation, an average of 34% of the DCs in the spleen and 25% in the lymph nodes (Fig. 1) expressed GFP. Thus, transduction of precursors, differentiation of transduced precursors into DCs and trafficking of DCs to secondary

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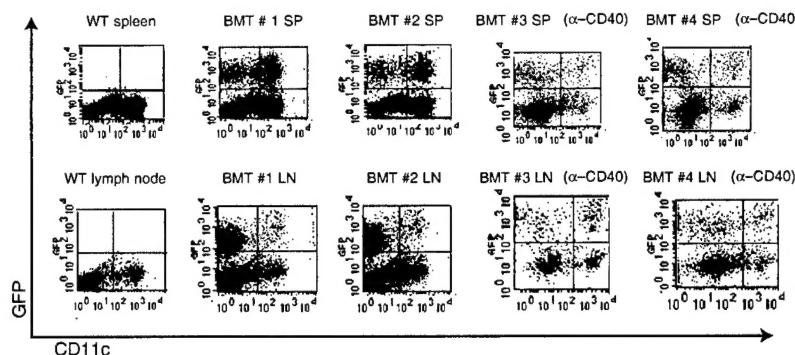


Figure 1 *In vivo* expression of transgene-encoded GFP by dendritic-cell progeny of transduced bone marrow stem-progenitor cells. DCs enriched from spleen and lymph nodes were analyzed by three-color FACS for MHC II, CD11c, and GFP (FL1) fluorescence. Shown are two representative plots from nontransplanted wild-type BALB/c mice (WT), two representative plots from transplanted mice not treated with antibody to CD40 (BMT #1 and #2) and two transplanted mice treated with antibody to CD40 (BMT #3 and #4), gated on live MHC II^{hi} cells. For the transplanted mice, four mice treated with antibody to CD40 and four untreated mice were analyzed in two separate experiments. WT, wild-type; SP, spleen; LN, lymph node; α -CD40, antibody to CD40.

lymphoid organs were all efficient. Transduction was not selective for DC progenitors, as similar percentages of transduced cells were obtained from the CD11c⁺ and lymphocyte fractions (data not shown).

Expansion of antigen-specific T cells after BMT

To determine whether successful expression of the transgene and trafficking of transduced DCs would result in activation of antigen-specific T cells, we conducted BMT with HSCs transduced with control and influenza hemagglutinin genes and analyzed antigen-specific immune responses using transgenic T cells specific for hemagglutinin. Expansion of hemagglutinin-specific CD4⁺ T cells (termed 'clone 6.5') and CD8⁺ T cells (termed 'clone 4') was used to assess priming of naïve T cells after adoptive transfer into recipients. Because the induction of T-cell tolerance to tumors adds an additional hurdle for immunotherapy, we analyzed T-cell proliferation in mice that were injected intravenously with

hemagglutinin-expressing A20 lymphoma (A20-hemagglutinin) cells 10 d before transplant. Mice were then transplanted with either hemagglutinin-transduced or control (GFP)-transduced HSCs, allowed engraftment time, then administered 2.5×10^7 total spleen cells from the clone 6.5 mice (10% of which are clone 6.5 transgenic CD4⁺ T cells). Spleens and lymph nodes were harvested 5 d after this adoptive T-cell transfer and analyzed for number and activation state. No significant expansion of clone 6.5 cells was observed as a result of antigen expression by tumor alone, or without systemic DC-activating agents. When Flt-3L and the agonistic antibody to CD40 were administered, however, a significant ($P = 0.02$) expansion and upregulation of CD44 by hemagglutinin-specific CD4⁺ T cells was achieved. Expansion of clone 6.5 CD4⁺ cells was dependent on transplantation of hemagglutinin-transduced HSCs. No significant expansion of hemagglutinin-specific CD4⁺ T cells was observed with control GFP-transduced BMT (Fig. 2a,b).

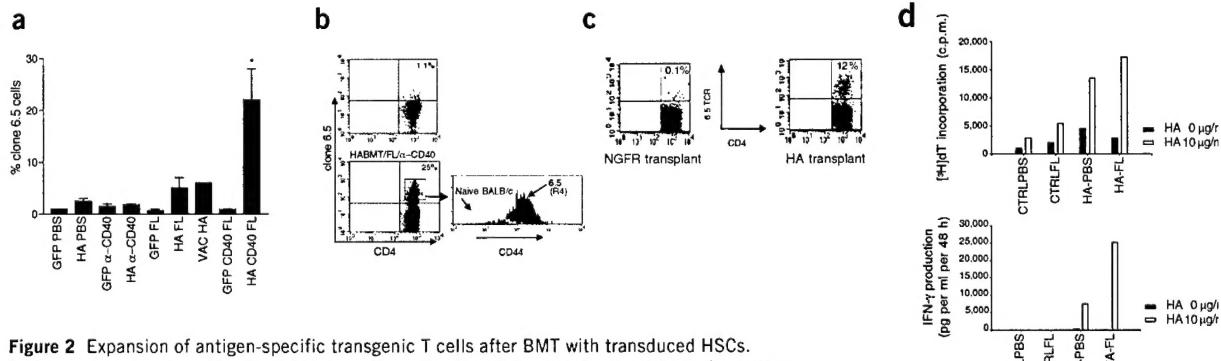


Figure 2 Expansion of antigen-specific transgenic T cells after BMT with transduced HSCs. (a) Proportion of splenic T cells staining positive with clone 6.5 monoclonal antibody, from mice treated with PBS, Flt-3L, antibody to CD40, Flt-3L plus antibody to CD40, or vaccinia expressing hemagglutinin. Shown are means \pm s.d. *, $P = 0.02$. (b) Representative FACS plot of control (top) and hemagglutinin transplant (bottom), both receiving Flt-3L + antibody to CD40. Also shown is the histogram of CD44 staining using naïve BALB/c as control, overlaid with expanded population of clone 6.5 cells (box 'R4' in upper right quadrant). (c) Long-term stability of gene expression in reconstituted mice. Shown is a FACS plot for CD4 and clone 6.5 TCR from lymph nodes from a control (transplanted with NGFR) and a hemagglutinin-transplanted mouse. (d) [³H]thymidine (dT) incorporation and IFN- γ production of splenocytes from mice receiving either control or hemagglutinin-transduced bone marrow and treated with either PBS or Flt-3L (FL). (e) Expansion of CD8 cells under optimal circumstances. Shown are representative FACS plots of a control transplant (left) and hemagglutinin transplant (right), with both groups receiving Flt-3L and antibody to CD40. The bar graph shows the average \pm s.d. of all mice in this experiment. *, $P = 0.008$. HA, hemagglutinin; α -CD40, antibody to CD40; FL, Flt-3L; VAC, vaccinia expressing hemagglutinin. NGFR, nerve growth factor receptor.

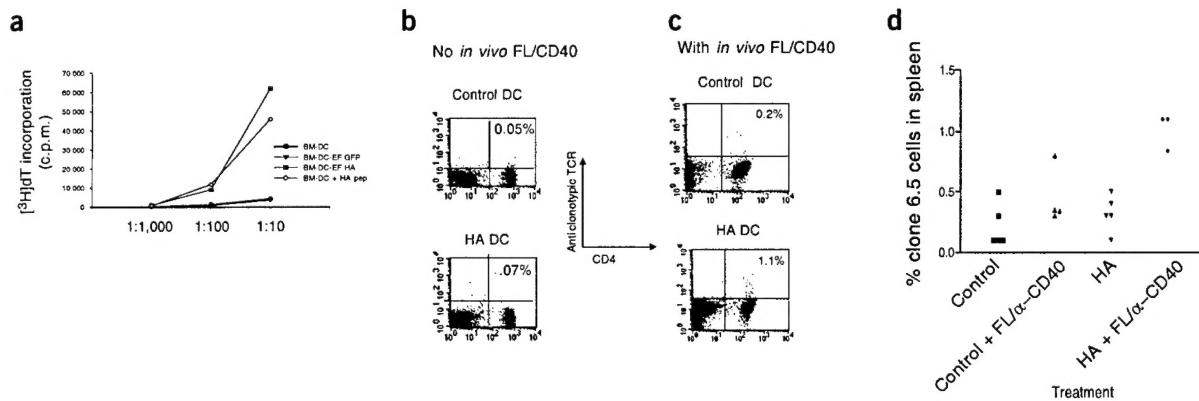


Figure 3 Immunization with *ex vivo*-transduced or loaded DCs leads to minor expansion of hemagglutinin-specific clone 6.5 T cells *in vivo*. (a) [3 H]thymidine (dT) incorporation shows that *in vitro*-generated bone marrow DCs (BM-DC) induce a strong proliferative response in cognate T cells. (b, c) Analysis of clone 6.5 expansion in splenocytes of BALB/c mice immunized with hemagglutinin-transduced DCs, without (b) or with (c) Flt-3L (FL) and antibody to CD40 (α -CD40). Analyzed as in **Figure 2**. (d) Percentage of clone 6.5 T cells in the spleen of each mouse analyzed in this experiment.

To determine the durability of transgene expression after HSC transplantation, we transplanted naive mice with either control or hemagglutinin-modified HSCs and left them unmanipulated for 1 year. We then treated the mice with the standard regimen of Flt-3L and antibody to CD40, as well as DLI from clone 6.5 mice, then analyzed their spleens for expansion of clone 6.5 cells. A substantial expansion in antigen-specific T cells could be elicited even 1 year after transplant, indicating long-term functional expression of the gene (Fig. 2c).

Expanded T cells exhibit effector function

To verify that the hemagglutinin-specific CD4 $^+$ T cells had effector function, proliferation in response to antigen and secretion of interferon (IFN)- γ were evaluated in culture after stimulation with hemagglutinin peptide. Spleen and lymph node cells were cultured with 0 or 10 μ g/ml hemagglutinin peptide for 48 h, and the supernatant was analyzed by ELISA for IFN- γ production. Proliferative response was assessed by [3 H]thymidine incorporation. Expanded hemagglutinin-specific cells proliferated in response to antigen and secreted IFN- γ , as shown, indicating that they were responsive to antigen (Fig. 2d).

To determine whether CD8 $^+$ T cells would be activated under the optimal conditions for expanding CD4 $^+$ T cells, the same procedure was used, except that the adoptive transfer involved hemagglutinin-specific transgenic CD8 $^+$ T cells. Both the control antigen-modified and the hemagglutinin gene-modified BMT groups received Flt-3L and antibody to

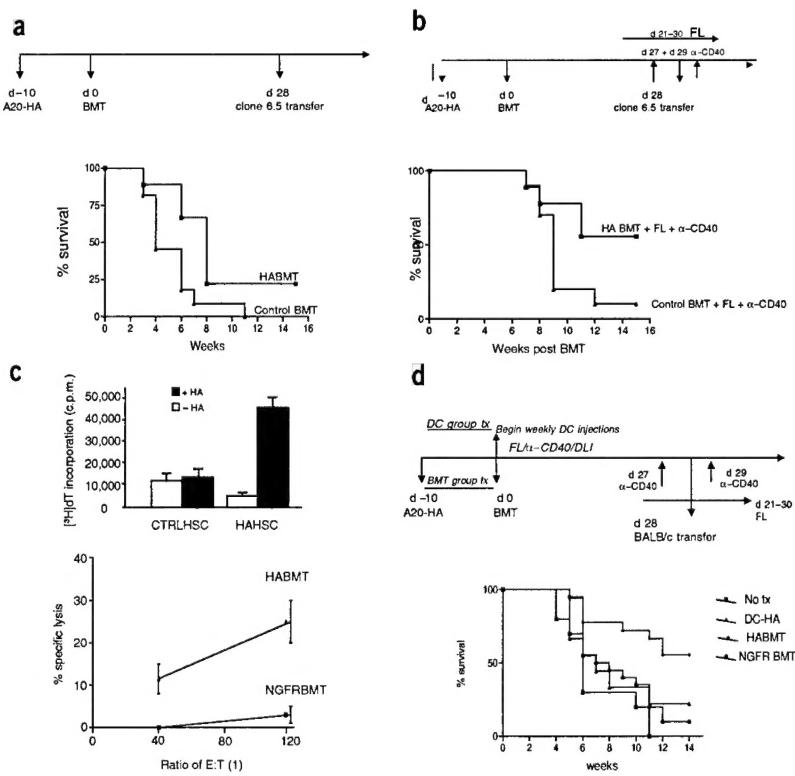


Figure 4 Treatment of A20-hemagglutinin tumor-bearing mice²³ with hemagglutinin-transduced HSCs and hemagglutinin-transduced DCs. (a) Survival of mice transplanted with control or hemagglutinin-transduced HSCs, DLI, and clone 6.5 splenocytes. $P = 0.1$. (b) Survival of mice treated as in a, except with Flt-3L and antibody to CD40 along with the second DLI. $P = 0.04$. (c) [3 H]thymidine (dT) incorporation and CTL of splenocytes from the surviving control and hemagglutinin mice from b, cultured either with (■) or without (□) hemagglutinin (HA) peptide. Specific lysis without peptide was subtracted from percent lysis with peptide for mice receiving hemagglutinin BMT ($n = 6$) or control (NGFR) BMT ($n = 2$). (d) Survival of mice subjected to the regimen indicated (either BMT or hemagglutinin-transduced DCs) as therapy (tx). Results are shown pooled from two separate experiments. $P = 0.04$ for HA BMT versus HA DCs.

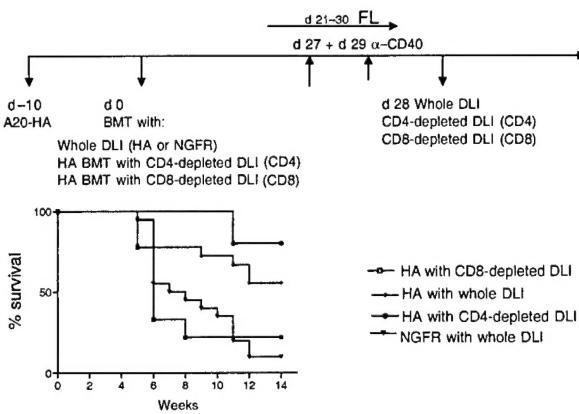
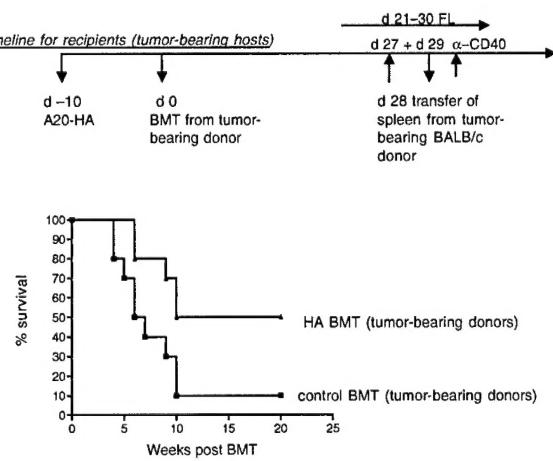


Figure 5 Antitumor immunity from hemagglutinin-transduced bone marrow is dependent on post-transplant administration of CD8⁺ T cells. Survival of mice treated with either CD4- or CD8-depleted DLI, as indicated. $P = 0.03$ for whole DLI versus CD8-depleted. FL, FH-3L; HA, hemagglutinin; α -CD40, antibody to CD40.

Initially, expression of antigen in the HSCs with no systemic agents was tested for antitumor effect. Mice were inoculated with tumor cells, transplanted with transduced HSCs, allowed to engraft and then given clone 6.5 cells but no Flt-3L or antibody to CD40. In the absence of systemic DC activators, there was a delay in tumor progression relative to mice receiving control transduced HSCs, but almost all mice ultimately succumbed to tumor, and no statistical significance was shown by a Kaplan-Meier survival analysis (Fig. 4a).

To assess whether systemic DC activators enhanced antitumor efficacy, additional groups of mice were also administered Flt-3L and antibody to CD40. Tumor was administered 10 d before BMT. Mice were transplanted on day 0 with either control or hemagglutinin-expressing HSCs, allowed to engraft, then given systemic Flt-3L on days 21–30, with 2 injections of antibody to CD40 on days 27 and 29 and DLI containing clone 6.5 T cells on day 28. Under these conditions, Flt-3L and antibody to CD40 prolonged survival, even in mice receiving control HSCs. Nearly all these mice eventually died, however. In contrast, over 50% of the mice receiving hemagglutinin-transduced HSCs and Flt-3L plus antibody to CD40 were alive at the termination of the experiment, more than 4 months later (Fig. 4b). Both of the remaining control mice alive at the termination of the experiment had gross signs of tumor at autopsy, whereas none of the hemagglutinin-transplanted survivors did.

To assess the persistence of hemagglutinin-specific T-cell activity, spleen cells from the surviving mice shown in Figure 4b were stimulated in culture with the cognate MHC class II-restricted hemagglutinin peptide and evaluated for proliferation. Whereas spleen cells from the remaining mice receiving control HSCs did not proliferate, a significant ($P = 0.03$) hemagglutinin-specific proliferative response was observed for the cells from the hemagglutinin transplants (Fig. 4c). Spleen cells from surviving mice were also analyzed for CTL activity against hemagglutinin-pulsed targets. Figure 4c shows the comparison of peptide-specific lysis by spleen cells from mice receiving control versus hemagglutinin-transduced HSCs. Mice that received BMT with hemagglutinin-expressing HSCs showed enhanced hemagglutinin-specific lysis compared with control transplanted mice.



CD40. DLI contained 2.5×10^7 spleen cells from clone 4 mice. A significant ($P = 0.008$) expansion was observed with CD8⁺ cells in the mice receiving hemagglutinin-modified HSCs. Because no antibody for the clone 4 T-cell receptor (TCR) was available, staining was done with antibody to V β 8.2, the V β region of clone 4 T cells. In mice transplanted with hemagglutinin-transduced HSCs, V β 8.2-positive cells expanded to ~30%, compared with the baseline 15% endogenously occurring in control transplanted mice (Fig. 2e). Thus, this procedure efficiently stimulates antigen-specific CD8⁺ cells *in vivo*.

We sought to assess the relative efficacy of T-cell stimulation by *ex vivo*-generated DCs, compared with *in vivo*-expanded DCs from hemagglutinin-transduced HSCs. We generated DCs from bone marrow cultures, transduced them with control or hemagglutinin vectors, and evaluated their *in vitro* and *in vivo* stimulatory capacities. For *in vitro* studies, DCs were plated with responding clone 6.5 spleen cells. For *in vivo* studies, mice were injected subcutaneously with 1×10^6 DCs, 2 d before and 2 d after the clone 6.5 transfer. DCs transduced with the hemagglutinin gene or pulsed with hemagglutinin peptide stimulated a robust *in vitro* T-cell response (Fig. 3a) but did not stimulate significant *in vivo* expansion of clone 6.5 T cells in either spleen (Fig. 3b) or lymph nodes (data not shown). The same experiment was conducted with the addition of systemically administered Flt-3L and antibody to CD40 to determine whether activation of injected DCs would increase their stimulatory capacity. These agents increased the expansion of clone 6.5 cells, but to an order of magnitude lower than the combination of hemagglutinin-transduced HSC transplantation, Flt-3L and antibody to CD40 (Fig. 3c). The percentage of clone 6.5 cells present in the spleen of each mouse analyzed is shown in Figure 3d. Taken together, these results show that the combination of transplantation of hemagglutinin-transduced HSCs and systemic administration of DC activators is a potent means of expanding antigen-specific T cells *in vivo*.

Transduced HSCs as a therapy for established tumors

We next sought to determine whether the increased T-cell stimulation by antigen-transduced HSCs imparted an enhanced therapeutic effect against established tumors. Mice were injected with A20-hemagglutinin cells 10 d before the initiation of therapy, followed by transplantation, as shown in the schema (Fig. 4a,b,d), then evaluated for survival.

Figure 6 Treatment of tumor-bearing mice with hemagglutinin-transduced bone marrow from tumor-bearing donors. Survival of mice treated as indicated. $P = 0.026$. FL, FH-3L; HA, hemagglutinin; α -CD40, antibody to CD40.

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Because the transgenic DLI creates an artificially high precursor frequency of antigen-specific cells, the experiment was repeated with the same schema as above, except that the DLI was derived from wild-type BALB/c mice. We also compared the efficacy of our transduced HSC strategy with 'classic' DC vaccination using *ex vivo*-generated, lentivirally transduced DCs. All mice (ten per group) were inoculated with A20-hemagglutinin cells 10 d before the initiation of therapy. The non-treated group was left unmanipulated. On day 0, the DC vaccine group began receiving subcutaneous immunization of hemagglutinin-transduced, *ex vivo*-generated DCs and the regimen of Flt-3L, antibody to CD40 and DLI. This group received weekly injections of transduced DCs, which were generated and tested every week for *in vitro* stimulatory capacity of clone 6.5 transgenic T cells before infusion. The BMT group was transplanted on day 0, allowed to engraft for 3 weeks, and treated with Flt-3L, antibody to CD40 and DLI. Mice were then observed for survival. Mice receiving DCs transduced with hemagglutinin and activated *in vivo* had prolonged survival compared with the untreated group, but had significantly ($P = 0.04$) shorter survival than those transplanted with hemagglutinin-transduced HSCs (Fig. 4d).

A significant ($P = 0.003$) enhancement in survival occurred in the mice transplanted with hemagglutinin-expressing HSCs and receiving naïve BALB/c DLI, similar to the previous experiment in which mice received DLI containing clone 6.5 cells (Fig. 4c). Thus, although inclusion of the transgenic clone 6.5 cells allows tracking of the activation of an antigen-specific response, transgenic cells were not necessary for tumor regression.

CD8⁺ T cells are required for the antitumor effect

Preliminary results showed that DLI was necessary for the antitumor effect. We therefore analyzed the cellular-fraction of DLI that was required. A20-hemagglutinin tumor-bearing mice were transplanted with hemagglutinin-expressing HSCs, treated with Flt-3L plus antibody to CD40, then provided with DLI of (i) whole spleen, (ii) spleen depleted of CD4⁺ cells or (iii) spleen depleted of CD8⁺ cells, or transplanted with control HSCs and whole spleen, as shown in Figure 5. CD4⁺ cells were not required for the effect. Moreover, depletion of CD4⁺ cells produced a trend toward enhanced survival, whereas depletion of CD8⁺ cells eliminated the therapeutic effect (Fig. 5).

Therapy with tumor-bearing donors as model for autologous BMT
Because autologous BMT is important for this therapy, we tested its efficacy in a setting in which the HSC and DLI donors had pre-existing tumors. Donor mice were injected with A20-hemagglutinin 3 weeks before collection of bone marrow and lymphocytes. Otherwise, the regimen was the same as in Figure 4, with experimental mice injected with A20-hemagglutinin 10 d before BMT, transplanted with either control or hemagglutinin-modified HSCs, and treated with Flt-3L, antibody to CD40 and DLI from tumor-bearing donors. Mice receiving hemagglutinin-transduced HSCs and DLI from tumor-bearing donors showed significantly ($P = 0.026$) enhanced survival compared with mice receiving control HSCs (Fig. 6).

DISCUSSION

Our results show that a potent antigen-specific immune response can be generated by expression of the antigen in DCs derived from transduced HSCs used for transplantation and then differentiated *in vivo*. This immune response led to long-term survival in a tumor therapy model. We evaluated antigen-specific therapy strategies after tumors had been established for 10 d, a period during which antigen-specific tolerance is established²¹. Profound hemagglutinin-specific tolerance is generated within 7 d after intravenous tumor injection, after which

standard therapeutic vaccination protocols are unable to successfully treat tumor-bearing animals. In our system, the combination of BMT with hemagglutinin-transduced HSCs, activation of antigen-presenting cells (APCs) by systemic agents, and provision of mature lymphocytes to respond to the activated DCs imparted a significant advantage over *ex vivo*-generated, transduced DCs administered after tumor establishment. Expression of antigen by the DCs induced proliferation and IFN- γ production by the responding T cells, indicating that these T cells were activated and retained effector function. The post-BMT setting favors enhancement of tumor immunity as well, for a number of reasons, including the potential for redirecting the immune system toward specific antigens²².

Our tumor therapy is likely to be dependent on efficient expression of the antigen in DCs in lymphoid compartments. We measured the effectiveness of antigen presentation *in vivo* by determining the expansion of antigen-specific T cells after BMT. Although the tumor itself expresses hemagglutinin, no expansion of antigen-specific T cells occurred with tumor alone, even after treatment with DC activators. Our previous findings showed an endogenous activation of tumor-specific T cells when lymphocytes are transferred on the day of transplantation into irradiated, tumor-bearing recipients²³, but this is probably a result of the effects of radiation-induced lymphopenia, tumor antigen release and APC activation in the early post-transplant period, all of which favor T-cell activation rather than tolerance. In contrast, delayed T-cell transfer into mice with residual tumors (as in the current study) leads to tolerance. We tested whether the process of gene-modified BMT and systemic DC activation could lead to activation of transferred antigen-specific T cells in the presence of this tolerizing environment, and found a substantial expansion of both CD4⁺ and CD8⁺ cells. In animals transplanted with hemagglutinin-transduced HSCs and treated with systemic DC activators, hemagglutinin-specific T-cell activation dominated over tolerance induction. In this setting, clone 6.5 cells expanded by 2 logs and acquired effector function. This level of expansion of hemagglutinin-specific CD4⁺ T cells *in vivo* was not observed after immunization with either hemagglutinin gene-transduced or hemagglutinin peptide-loaded DCs, even after *in vivo* activation with Flt-3L and antibody to CD40, although improved expansion could possibly be achieved under optimal DC-activating conditions. Additional improvements in DC vaccines may lead to enhanced efficacy if they are effectively matured and processed, as evidenced by recent studies showing that enhanced responses were obtained with tumor-pulsed DCs in the context of BMT¹⁵ and also with *in vivo* activation of DCs to present an antigen introduced by vaccination²⁴.

In our system, Flt-3L and antibody to CD40 prolonged survival of both control and hemagglutinin-transplanted mice, which is consistent with previous studies showing that Flt-3L and CD40 antibody treatment alone stimulated an antitumor response²⁵. Flt-3L generates large numbers of APCs and has been used both *ex vivo* and *in vitro* to produce DCs¹⁹. Activation through CD40 produces crucial events such as upregulating costimulatory molecules, converting tumor-specific tolerance to priming²⁰ and enhancing the efficacy of both vaccines¹⁹. In our study, provision of DLI after APC activation may contribute to activation rather than tolerance of the DLI, as immature DCs can tolerate T cells²⁶.

The goal of analyzing transgenic T cells is to show activation of antigen-specific cells. The clone 6.5 T cells allow tracking of antigen-specific responses but are not required for antitumor efficacy, and mobilization of antigen-specific cells at a lower precursor frequency produced a similar effect. In preliminary studies not shown here, administration of DLI was required for an antitumor effect. Our cur-



rent results addressed the component of DLI necessary for the effect, in experiments using DLI depleted of either CD4⁺ or CD8⁺. Depletion was conducted for DLI given both at transplant and at the later time point. Depletion of CD8⁺ T cells substantially decreased survival, whereas depletion of CD4⁺ T cells did not. In fact, there was a trend toward enhanced survival with DLI depleted for CD4⁺ cells, although it was not significant. It is possible that regulatory T cells dampen the response, but further studies are necessary to investigate this finding. Activation of the CD8⁺ T cells is likely to be at least partly responsible for the observed antitumor effect.

Taken together, our findings suggest a new approach to the induction of potent antigen-specific immunity using antigen gene-transduced HSCs capable of curing animals with established tumors. Translation of this approach to the clinic will require effective transduction of human DC progenitors in the context of BMT, for cancers with known antigens. Thus, application may be limited to a select group of diseases at present, and will require validation of the approach.

METHODS

Animals. BALB/c mice (4–8 weeks old) were purchased from the National Cancer Institute. Clone 6.5 (hemagglutinin-specific CD4⁺ TCR-transgenic) mice were initially obtained from H. von Boehmer (Harvard University, Boston, Massachusetts); clone 4 (transgenic hemagglutinin-specific CD8⁺ TCR) mice were obtained from L. Sherman (Scripps Institute, La Jolla, California) and bred and housed at Johns Hopkins Medical Institute (JHMI); all were maintained under protocols approved by JHMI.

Lentiviral vector construction and virus production. The vector Sin-18 (PGK.GFP) was provided by D. Trono (University of Geneva, Switzerland). EGFP and EF hemagglutinin were constructed by replacing the PGK promoter with the human elongation factor EF-1 α promoter. All inserts were confirmed by sequencing. Vector supernatants were produced and concentrated as described²⁷. The nerve growth factor receptor (NGFR) gene was also used as a control insert for some experiments¹⁶.

Antibodies and flow cytometry analysis. R-phycocerythrin-conjugated antibodies to CD11c, V β 8.2 and I-E κ ; cychrome-conjugated antibodies to CD4 or CD8; and FITC-conjugated antibody to CD44 were purchased from PharMingen. The clone 6.5 antibody has been described²³. FACS analysis was carried out using a Becton Dickinson FACScan.

Mouse bone marrow harvesting, lineage depletion, and transduction. All samples used for BMT were enriched for lineage antigen-negative (Lin^{Neg}) cells using the StemSep mouse progenitor enrichment kit (StemCell Technologies). In all transplants, the isolated bone marrow that was enriched for Lin^{Neg} cells (referred to throughout as HSCs) was used. This enrichment depletes >97% of lineage-specific cells. For transduction, these cells were cultured overnight in Quality Biologics Serum Free (Quality Biologics) with 100 ng/ml mouse stem cell factor, 10 ng/ml thrombopoietin, and 50 ng/ml Flt-3L (Peprotech) before gene transfer. Three rounds of transduction were conducted by adding concentrated supernatant at an MOI of 2–5 in the presence of 8 μ g/ml polybrene.

In vitro differentiation of lentivirally transduced mouse bone marrow cells and HSCs. Bone marrow was differentiated into DCs by culturing the transduced cells in RPMI-5 in the presence of mouse granulocyte-macrophage colony-stimulating factor (1,000 U/ml) for 8 d, following conventional protocols. Mature DCs in suspension were harvested on day 8 and used for FACS analysis and functional assays. Class I-restricted (amino acids 518–526) and Class II-restricted (amino acids 110–120) hemagglutinin peptides were obtained from Macromolecular Resources. IFN- γ production was determined by ELISA (Pierce-Endogen) following the manufacturer's instructions.

BMT and analysis of transgene expression. Three days after the transduction, 10⁵ transduced Lin^{Neg} cells were collected and transplanted intravenously into irradiated (850 cGy) BALB/c mice. Transduction efficiency of this approach ranges from 10–15% expression *in vitro* before transplantation²⁷, with addi-

tional expansion *in vivo* after transplantation ranging from 20–35% (Fig. 1). Splenic and lymph node dendritic cells were prepared by adhering collagenase-treated splenocytes in 6-well plates at 37 °C for 3 h, followed by overnight culture.

For tumor therapy studies, 10⁶ A20-hemagglutinin cells (from a mouse B-cell lymphoma modified to express hemagglutinin²³) were injected 10 d before BMT, with 8–12 mice per group. Flt-3L (Peprotech) was injected subcutaneously at 10 μ g per mouse per d for 10 d. Antibody to CD40 (FGK4.5) was injected intravenously at 100 μ g per mouse per d, on days 7 and 9 of the Flt-3L treatment. For adoptive T-cell transfer, splenocytes were harvested from BALB/c, clone 4 or clone 6.5 mice (2.5 \times 10⁷ total splenocytes were infused). Statistical analysis was conducted with GraphPad software. CD4 and CD8 depletion was conducted with MACS bead systems (Miltenyi) according to the manufacturer's instructions.

Hemagglutinin-specific T-cell responses to transduced DCs. Spleen and lymph nodes of clone 6.5 and BALB/c mice were collected, and T lymphocytes were nylon wool-enriched and seeded in 96-well plates at 2 \times 10⁵ cells/well. Hemagglutinin-expressing bone marrow DCs derived from 8-d culture of EF hemagglutinin-transduced bone marrow cells, along with mock-transduced bone marrow DCs, were irradiated at 3 Gy and seeded in the 96-well plates with T cells in triplicates at the stimulator (DC)-to-effector (T cells) ratios shown in Figure 4c. Three days later, the wells were pulsed with 1 μ Ci/well [³H]thymidine and harvested 18–20 h later with a Packard Micromate cell harvester. The ³H incorporation was determined as counts per minute through a Packard Matrix 96 direct beta counter.

CTL analysis. In a JAM test²⁸, spleen cells from experimental mice were collected and plated at 4 \times 10⁶ cells/well in a 24-well dish with class I peptide at 5 μ g/ml and interleukin-2 at 10 U/ml. Three days later, a BALB/c spleen was harvested and plated at 2 \times 10⁶ cells/well in 24 wells with ConA (Sigma) at 2 μ g/ml. Two days after that, the BALB/c targets were pulsed with 5 μ Ci [³H]thymidine per well. After an overnight incubation, half the targets were pulsed for 1–2 h with 10 μ g/ml class I peptide and half were left unpulsed. Targets were then harvested, washed and incubated with experimental spleens for 4–6 h, and specific lysis was calculated.

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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